Contamination is a Frequent Confounding Factor in Toxicology Studies with Anthraquinone and Related Compounds

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Abstract

Anthraquinone (AQ) (9,10-anthracenedione) is an important compound in commerce. Many structurally-related AQ derivatives are medicinal natural plant products. Examples include 1-hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ) which are also metabolites of AQ. Some commercial AQ is produced by the oxidation of anthracene (AQ-OX). In the recent past, the anthracene used was distilled from coal tar and different lots of derived AQ often contained polycyclic aromatic hydrocarbon contaminants, particularly 9-nitroanthracene (9-NA). Many toxicology studies on AQ used contaminated anthracene-derived AQ-OX, including a National Toxicology Program (NTP) two-year cancer bioassay which reported a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and in the livers of male and female B6C3F$_1$ mice. The AQ-OX used in that bioassay was mutagenic and contained 9-NA and other contaminants. In contrast, purified AQ is not genotoxic. The purpose of this paper is to provide additional information to help iterpret the NTP cancer bioassay. This paper describes a quantitative analytical study of the NTP anthracene-derived AQ-OX test material, and presents the results of mutagenicity studies with the 1-OH-AQ and 2-OH-AQ metabolites and the primary contaminant 9-NA. Purified 1-OH-AQ and 2-OH-AQ exhibited only weak mutagenic activity in selected strains of tester bacteria and required S9. Literature reports of potent mutagenic activity for 1-OH-AQ and 2-OH-AQ in bacteria minus S9 are, once again, very likely the result of the presence of contaminants in the test samples. Weak activity and limited production of the 1-OH-AQ and 2-OH-AQ metabolites are possible reasons that AQ fails to exhibit activity in numerous genotoxicity assays. 9-NA was mutagenic in tester strains TA98 and TA100 minus S9. This pattern of activity is consistent with that seen with the contaminated AQ-OX used in the NTP bioassay.

Analysis of all the mutagenicity and analytical data, however, indicates that the mutagenic contamination in the NTP bioassay probably resides with compounds in addition to 9-NA. 9-NA exhibited potent mutagenic activity in the L5178Y mammalian cell mutagenicity assay in the presence of S9. The positive response was primarily associated with an increase in small colony mutants suggesting a predominance of a clastogenic mechanism. Quantitative mutagenicity and carcinogenicity potency estimates indicate that it is plausible that the contaminants alone in the NTP AQ-OX bioassay could have been responsible for all of the observed carcinogenic activity. Although AQ-OX is no longer commercially used in the United States, many of the reported genotoxicity and carcinogenicity results in the literature for AQ and AQ derivative compounds must be viewed with caution.

Keywords Anthraquinone, Contamination Issues, 1-Hydroxyanthraquinone, 2-Hydroxyanthraquinone, Mutagenicity

Introduction

Anthraquinone (AQ) (9,10-anthracenedione) and structurally-related compounds are important in commerce and many are found as natural plant products. AQ is used as an intermediate in the manufacture of dyes and to enhance the efficiency of the Kraft Process for the production of paper, thus reducing the number of trees harvested (Cofrancesco, 1992). AQ is the active ingredient in the most effective and nonharmful bird repellent used for keeping birds from airport runways or areas where they would conflict with the human population (Ballinger and

Price 1996; Cummings et al. 1997; Ballinger et al. 1998; Dolbeer et al. 1998). AQ derivatives are widely found in plants. Hydroxyanthraquinones are the biologically active components of many phytotherapeutic drugs including plant-derived laxatives such as aloe, senna, frangula, emodin, and rheum. Uses also include treatments for kidney and bladder stones, and as a mild sedative (Tikkanen et al. 1983; Westendorf et al. 1990).

Reports of the activity of AQ and related compounds in genetic toxicology and carcinogenicity assays are decidedly mixed, with many examples of opposite results reported from different laboratories. A critical review of the literature shows that in many cases, contamination with mutagenic compounds is the probable reason for the contrasting results. It is common that the focus of a publication will be on one or a set of anthraquinone derivatives found naturally in plants, yet the test material for the genetic toxicology or cancer studies will be a synthetic product with the potential for containing biologically active contaminants (Tikkanen et al. 1983; Kawai et al. 1986, Westendorf 1990; Mori et al. 1990, Mori et al. 1992, NTP 2004).

From a toxicological viewpoint, a recurring problem in studies reported earlier is that a formerly common synthetic pathway to produce AQ was from the oxidation of anthracene (AQ-OX). The anthracene used was distilled from coal tar and different lots of derived AQ contained varying amounts of polycyclic aromatic hydrocarbon contaminants, particularly the mutagenic isomers of nitroanthracene (Cofrancesco, 1992; Butterworth et al. 2001). In a two year study the National Toxicology Program (NTP) reported that anthracene-derived AQ-OX induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a modest

increase in the livers of male and female B6C3F$_1$ mice (NTP, 2004). Further analysis of that study showed that the anthracene-derived test material was contaminated with 9-nitroanthracene as well as other polycyclic aromatic hydrocarbons and was also mutagenic in bacterial assays (NTP, 2004; Butterworth et al. 2001). When the test material was purified, the pure AQ was found to be without mutagenic activity (Butterworth et al. 2001). In contrast to most reports, however, the NTP noted that the study material was anthracene-derived AQ-OX and acknowledged the contamination issue. Thus, while the NTP bioassay is a valid study that directly applies to exposure to AQ-OX, the degree to which pure AQ may be carcinogenic is not known. The main purpose of the analytical and genotoxicity studies reported in this paper was to provide additional information to help with the interpretation of that NTP bioassay. In the course of this evaluation, it became evident that mutagenicity and carcinogenicity studies with some of the AQ derivatives were also likely affected by contamination as a result of the particular synthetic pathways used in their preparation.

In assessing the potential biological activity of preparations of AQ, it is critical to be aware of how the preparation of interest was manufactured and the potential contaminants inherent with the different synthesis processes. AQ is produced in large quantities by three different production methods in various parts of the world (Cofrancesco 1992). The oxidation of anthracene to yield AQ is practiced primarily in Europe, but is now in declining use. AQ from the oxidation process (AQ-OX) involves the oxidation of anthracene derived from coal tar. Distillates that carry-over in the same fraction as anthracene often remain as contaminants in the final anthracene-derived AQ-OX product. These include a variety of polycyclic aromatic hydrocarbons with similar boiling points. For example 9-nitroanthracene and anthracene distill within 5 degrees of each other.

The AQ-OX contamination problem has been known for some time and profiles of contaminants from this process can differ substantially. Of particular concern is the observation that the mutagenic nitroanthracenes are often seen in AQ-OX preparations, sometimes at concentrations over 2.5% (U.S. EPA 1977; ICI 1978a, 1978b). An illustration of the variability between lots is illustrated by comparing the anthracene-derived AQ-OX used in the NTP cancer bioassay to that of the anthracene-derived AQ-OX used in the companion NTP mutagenicity studies (NTP 2004). The actual material used in the two-year cancer bioassay has a reported contaminant level in the range of 0.1 to 0.65%, depending on the analytical testing laboratory, and is a bacterial mutagen. Material used in the companion NTP mutagenicity studies had a contaminant level of 3% and was highly mutagenic. In both cases, when the anthracene-derived AQ-OX was purified and retested, no mutagenic activity was observed for the pure AQ (Butterworth et al. 2001; NTP 2004).

Benzene and phthalic anhydride undergo the Friedel-Crafts reaction to yield o-benzoylbenzoic acid, which is treated with concentrated sulfuric acid to yield AQ (Cofrancesco 1992). This is the most prevalent production method employed in China and India. AQ produced by the Friedel-Crafts process (AQ-FC) is not a bacterial mutagen and is substantially free of the polycyclic aromatic contaminants and nitroanthracenes that are often found in AQ-OX (Butterworth et al. 2001).

Production of AQ by the Diels-Adler reaction (AQ-DA) between 1,4-naphthoquinone and 1,3-butadiene is practiced primarily in Japan. Because this process involves shifts between the aqueous and organic phases, contaminants are

easily removed and AQ-DA is particularly clean and free of contaminants and is not mutagenic in a variety of genetic toxicology test systems (Butterworth et al. 2001).

All anthraquinone used commercially in the United States today is AQ-FC or AQ-DA, rather than AQ-OX. Unfortunately, chemical supply houses such as Zeneca Fine Chemicals and Aldrich Chemical Co. regularly stocked anthracene-derived AQ-OX at the time that the studies were begun by the NTP.

A revealing demonstration as to the variability in purity of different preparations of AQ with corresponding opposite conclusions as to genotoxic activity is to critically examine the published literature for this compound. Negative results in the Ames *Salmonella* bacterial mutagenicity assay have been reported by 9 independent laboratories (Brown and Brown 1976; Anderson and Styles 1978; Gibson et al. 1978; Salamone et al. 1979; Sakai et al. 1985; Tikkanen et al. 1983; National Cancer Institute 1987; Butterworth et al. 2001, NTP 2004). In contrast, positive mutagenicity assays were reported from two laboratories that used anthracene-derived AQ-OX (Liberman et al. 1982; Zeiger et al. 1988). The pattern of activity of AQ reported from the AQ-OX studies was also unusual in that mutagenic activity was seen without metabolic activation. The chemical structure of AQ does not suggest that the parent compound would be a DNA reactive mutagen. Comparative studies show that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism (Butterworth et al. 2001, NTP 2004).

Similar conflicting results were observed with micronucleus assays. AQ-DA tested negative in the *in vivo* mouse micronucleus assay (Butterworth et al. 2001). In

contrast, using contaminated anthracene-derived AQ-OX, the NTP reported a weak positive response in a mouse peripheral blood micronuclei assay test from the 14-week range finding study that preceded the cancer bioassay (NTP 2004). Even though the doses used were up to 4 times of the maximum tolerated dose used in the bioassay, no response was seen in the female animals, and the response in the males was judged as positive only with a highly nonconservative trend test. In contrast, anthracene-derived AQ-OX administered by intraperitoneal injection was negative in a bone marrow micronucleus assay (NTP 2004). Similarly, there is one report of weak induction of micronuclei in SHE cells, but the material used was again the contaminated NTP anthracene-derived AQ-OX (Gibson et al. 1997). The experimental evidence indicates that pure AQ does not induce micronuclei.

A characteristic of a nongenotoxic compound is demonstrated lack of activity in a variety of *in vivo* and *in vitro* genetic toxicology assays with different endpoints. In addition to testing negative in bacterial mutagenesis assays and the *in vivo* mouse micronucleus assay, AQ tested negative in the CHO chromosomal aberration assay (Butterworth et al. 2001), the L5178Y mouse lymphoma forward mutation assay (Butterworth et al. 2001), an assay assessing mutagenic activity in a line of human B-lymphoblastoid cells that constitutively express cytochrome P4501A1 (Durant et al. 1996), and in the Syrian hamster embryo (SHE) cell transformation assay (Kerckaert et al. 1996). This extensive testing battery clearly demonstrates that pure AQ is not genotoxic.

1-Hydroxyanthraquinone (1-OH-AQ) and 2-hydroxyanthraquinone (2-OH-AQ) are metabolites of AQ (NTP, 2004). 1-OH-AQ is present in some plant preparations as

the glycoside. Blomeke et al. (1992) reported that 1-OH-AQ was positive in the Ames mutagenicity bacterial tester strain TA1537 without S9. In contrast, the NTP found 1-OH-AQ to be without mutagenic activity in the bacterial mutagenicity assays they conducted (NTP, 2004). The NTP, however, reported that 2-OH-AQ was a potent bacterial mutagen in strain TA98 without S9 (NTP, 2004). Of concern with these studies is that neither 1-OH-AQ nor 2-OH-AQ appear to have the structure of a direct acting DNA-reactive mutagen and, thus, reports of activity without S9 are suspect. Mori *et al.* (1990) found that 1-OH-AQ induced tumors in the large bowel, liver, and stomach of treated rats. Kawai et al. (1986) reported that 1-OH-AQ induced DNA repair in primary rat hepatocytes. Interestingly, mechanistic studies indicate that the 1-OH-AQ induced large bowel tumors are likely produced to a large extent via a nongenotoxic-cytotoxic mode of action resulting from biological activity associated with high-dose, chemically-induced severe inflammation, crypt abscesses and erosion, and ulcerative colitis in the colonic mucosa (Mori et al. 1992; Yoshimi et al. 1995, Butterworth et al. 1995). Thus, the degree to which 1-OH-AQ and 2-OH-AQ may be mutagens is not clear from these studies.

The above studies on 1-OH-AQ and 2-OH-AQ were conducted at a time when the use of anthracene based 1-OH-AQ and 2-OH-AQ was common and the purity of the compounds used is not clear in each case. The most probable route of manufacturing 1-OH-AQ and 2-OH-AQ was either beginning with coal tar derived AQ-OX or by a three step process from 1- and 2-nitroanthraquinone. Both routes provided ample opportunity for mutagenic polycyclic aromatic hydrocarbon contamination.

To further illustrate this concern, in the studies reported here with 1-OH-AQ and 2-OH-AQ, a commercial source of these compounds could not be found that was not

heavily contaminated (Materials and Methods). Thus, one purpose of the current studies was to evaluate the mutagenic activity of purified 1-OH-AQ and 2-OH-AQ to bring some clarity to the issue of mutagenic potential.

9-NA at 0.12% was identified as the most prevalent contaminant in the NTP anthracene-derived AQ-OX bioassay material (Butterworth et al. 2001). No further analytical work was done at that time. Subsequently, the question was raised as to whether the mutagenic activity of 9-NA alone was of sufficient potency to account for the degree of activity seen in the bioassay material. It was possible that the observed contaminating mutagenic and potential carcinogenic activity might reside with more than just the 9-NA. Accordingly, a precise quantitative analysis of the bioassay material was required. An NTP GC/FID analysis of the bioassay material indicated a contaminant level of 0.1% of 9-NA. However when the same material was evaluated using HPLC/UV analysis, a contaminant level of 0.5% was seen with two impurities of 0.3% and 0.2% relative to the AQ peak. The greater peak was identified as 9-NA. The second peak was not identified (NTP 2004). To resolve these discrepancies and provide needed analytical information, a more rigorous analytical evaluation of the NTP bioassay material was undertaken.

Nitroaromatic compounds can be extremely potent mutagens and carcinogens (Pitts et al. 1982, Fu et al. 1985; Fu et al. 1986; Durant et al. 1996). Uncertainty remained, however, regarding the biological activity of 9-NA. Was the mutagenic activity seen in the bioassay material completely accounted for by the 9-NA, or did the activity also reside with other contaminants? For example, 9-NA is only a modest bacterial mutagen while 2-NA is of such incredible potency to place it in the category of a super mutagen (Fu et al. 1986; NTP 2004). Only a trace amount of 2-NA would be required to contribute substantial mutagenic activity. Compounds such as 9-NA, 2-NA, and 2-nitrofluorene are active in bacterial assays without S9. Is such activity

without S9 applicable for mammalian cells? While bacterial mutagenicity is important, knowledge of genotoxic activity in mammalian cells may be more relevant. It had been reported that 9-NA was active in a mutagenicity assay in a human cell line expressing P450 1A1 (Durant et al. 1996). To address these questions, the activity of 9-NA was evaluated in the Ames bacterial mutagenicity assay as well as the mammalian cell mouse lymphoma mutagenesis assay.

MATERIALS AND METHODS

Chemicals

A sample of the AQ-OX powder employed in the NTP 2-year toxicology and carcinogenesis studies (NTP 2004) was generously provided by Cynthia Smith of the NTP and Donna Browning, NTP Chemical Custodian, Battelle, Columbus OH. This sample was designated as NTP AQ-OX. The bright yellow powder obtained from the NTP was labeled Anthraquinone, Battelle Task Identifier: 5-064-SHIP-211, lot: 5893, CAS: 84-65-1. The technical report stated that the sample had been analyzed by the NTP and was found to be about 99% anthraquinone and noted an impurity at a concentration of 0.12% (Battelle 1993; NTP 2004). Note that the AQ-OX used in the NTP companion mutagenicity studies had a contaminant level of 3% and was highly mutagenic (NTP 2004). The 9-nitroanthracene used was from Aldrich Chemical Co. and was 97% pure.

Analytical Analysis of the NTP Anthracene-derived AQ-OX Bioassay Material

The AQ-OX preparation that was used in the NTP bioassay (NTP, 2004) was studied using a rigorous analytical procedure specifically designed to quantitate impurities in

AQ-OX that can be missed by conventional techniques. GC analysis of AQ-OX can often fail to detect substantial contamination with low levels of multiple contaminants because the minimum amount of material is applied to the column to avoid overloading. A more thorough analysis may be obtained if the contaminants are removed and studied separately from the main material. An extraction process based on recrystallization from a solvent compatible with HPLC analysis was evolved. The extraction solvents used were ethanol and acetonitrile. They were chosen because they have a modest solubility for the AQ, will completely dissolve the expected organic impurities, have boiling points in the range of 75-85 degrees °C, have a density less than that of the solid AQ and are compatible with the mobile phase used in the HPLC analysis.

An accurately weighed sample of NTP AQ-OX between 0.030 and 0.064 g was transferred to a screwcap culture tube, 13 mm x 100 mm equipped with a Teflon lined cap. The solvent, 5.00 mL, was added to the tube and the cap was secured. The tube was placed in a heating block such that the solvent level was about 5-10 mm above the top of the block. The block was heated to 75 – 80 °C to dissolve the sample and to allow the crystals of the anthraquinone to form in the zone above the top level of the block. This zone was at a temperature below that in the lower part of the tube. Heating was continued for about 1.5 - 2 hr until the original sample was completely dissolved. When the original solids had been dissolved the tube was removed from the block and placed in a beaker to cool slowly. This combination of recrystallization during the dissolution process and the slow cooling allowed for separation of a high purity anthraquinone and left the original sample impurities in the clear solution. An aliquot of this solution was diluted with HPLC mobile phase, mixed and filtered through a 0.45 micron filter into a sample vial. The filtered solution was analyzed by HPLC using

a Zorbax C18 column operated in the reverse phase mode. A gradient elution program using acetonitrile and water was employed. The HPLC detector was run at 254 nm with data collected and processed using an electronic data system. The instrument response was calibrated with known standards.

Based on process knowledge for manufacture of AQ by oxidation of coal tar based anthracene, nitrobenzene, fluorenone and a certified standard of polyaromatic hydrocarbons (PAH) designated as EPA PAH 610 were purchased from Sigma Chemical (Milwaukee, WI) to provide calibration of the HPLC instrument for these compounds when present. The 9-NA standard was obtained from Fluka Chemical (Milwaukee, WI). The total method described above was validated by use of replicate samples of different weights, spikes of standard compounds added to the samples before extraction and added to the aliquots after extraction. The spike recovery for the 9-NA was consistently between 85 and 115% for spikes added to purified product at the level of 100 to 200 ppm and to sample extract at levels within the instrument calibration range. The minimum detection limit for the method was determined to be less than 2 ppm in the purified samples.

HPLC and GC detectors and configurations employed included HPLC/MS, GC/AP-MS, GC/MS, HPLC/UV, and HPLC/diode array. The HPLC/MS provided confirmation of the peaks observed in the routine HPLC/UV scans. The GC/AP-MS provided high sensitivity and specificity for determination of nitroaromatic compounds. The HPLC/diode array provided full spectra of the AQ and the 9-nitroanthracene. The detection limits for the sample impurities were significantly improved to values below 5 ppm for impurities such as 9-nitroanthracene, polycyclic aromatic hydrocarbons and other impurities of interest.

Purification of 1-OH-AQ and 2-OH-AQ for Mutagenicity Testing

No contaminant-free commercially-prepared sample of either 1-OH-AQ or 2-OH-AQ could be found. For example, an HPLC examination of a commercially produced lot of 2-OH-AQ showed that that preparation contained significant levels of multiple contaminants including anthrone, 1-chloroanthraquinone and 2-chloroanthraquinone. The contaminant level of the 1-chloroanthraquinone alone was 0.64%. The objective of these experiments was to examine the mutagenic activity of pure 1-OH-AQ and 2-OH-AQ. Therefore, obtaining pure test material was obligatory.

An attempt to purify the commercial 1-OH-AQ and 2-OH-AQ via a re-crystallization process from ethanol and acetonitrile failed to reduce the impurities by more than 50%. It was concluded that use of a commercial source and standard recrystallization techniques for 1-OH-AQ and 2-OH-AQ would not yield a product of sufficient purity to be used in the bacterial mutagenicity assays. Accordingly, it was decided that the 1-OH-AQ and 2-OH-AQ would have to be synthesized, purified, and analyzed in our laboratories using techniques that would yield a pure product.

1-OH-AQ and 2-OH-AQ was synthesized starting with purified amino-anthraquinones. The purified amino-anthraquinone was converted to the hydroxyanthraquinones by the classical diazotization of the amine bisulfate. The diazo salts were hydrolyzed to the hydroxyanthraquinones with sulfuric acid. The purification of 1-OH-AQ and 2-OH-AQ was done by extraction of the product with dichloromethane to separate it from amine compounds. The dichloromethane extract was washed with water, and then extracted with 1N sodium hydroxide in order to separate the product from impurities such as chloroanthraquinones and nitroaromatic compounds. The alkaline extract was acidified and the product was finally re-crystallized from ethanol to yield the final product. HPLC

analysis showed that the target impurities, amino and chloroanthraquinones and nitroaromatic compounds were below method detection limits of 5 ppm.

Bacterial Mutagenicity Assays

Samples of 1-OH-AQ, 2-OH-AQ, and 9-NA were submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the *Salmonella-Escherichia coli*/mammalian-microsome reverse mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Ames et al. 1975; Brusick et al. 1980; Maron and Ames, 1983). The assay assessed the ability of the test agent to induce mutations in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system. The activation system was a microsomal enzyme preparation derived from Aroclor induced rat liver (S9). Dose levels were based on a toxicity range finding study. The experiments were repeated independently to confirm initial results. 9-NA was evaluated in an abbreviated screening protocol using tester strains TA98 and TA100.

Criteria for a positive response were at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean revertants per plate of the appropriate vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a 3-fold increase for tester strains TA1535 and TA1537.

L5178Y TK +/- Mouse Lymphoma Forward Mutation Assay

A sample of 9-NA was submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the L5178Y TK+/- mouse lymphoma forward mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Amacher et al. 1980; Clive et al. 1987). The objective of the assay was to evaluate the ability of 9-NA to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. DMSO was used as the vehicle. At treatment termination, precipitate was observed in the treatment medium at concentrations from 20 to 1000 μg/ml. Assays were run with and without a rat liver S9 metabolic activation system. Range finding toxicity studies showed the testing limit to be 30 μg/ml without S9 and 50 μg/ml plus S9. The criteria for a positive response is induction of a mutation frequency that is at least two times that of the control mutant frequency for that given experiment. Colony sizing was done on cultures that produced a positive response with 9-NA.

RESULTS

Analytical Analysis of the NTP Anthracene-derived AQ-OX Bioassay Material

A sample of the AQ-OX preparation that was used in the NTP bioassay (NTP, 2004) was studied using a rigorous analytical procedure specifically designed to quantitate impurities in AQ-OX that can be missed by conventional techniques.

Analysis showed that the level of contamination in the bioassay material was 0.65%. The individual component in the highest amount was 9-nitroanthracene at a level of 0.11%. Other classes included polycyclic aromatic hydrocarbons at 0.09% (including anthracene,

phenanthrene, and dibenzo (a,h) anthracene); nitrobenzene at 0.05% and unidentified organics and nitro-organics at 0.40%.

Bacterial Mutagenicity of 1-OH-AQ

No commercial preparation of 1-OH-AQ was found that did not contain substantial contaminating material. Thus, 1-OH-AQ was synthesized and purified in our laboratory to meet a standard of less than 5 ppm contaminating material (Materials and Methods).

This pure sample of 1-OH-AQ was evaluated in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system (S9). The only activity observed for 1-OH-AQ was a weak response in TA1537 and required the presence of S9 (Table 1).

Bacterial Mutagenicity of 2-Hydroxyanthraquinone (2-OH-AQ)

No commercial preparation of 2-OH-AQ was found that did not contain substantial contaminating material. Thus, 2-OH-AQ was synthesized and purified in our laboratory to meet a standard of less than 5 ppm contaminating material (Materials and Methods).

This pure sample of 2-OH-AQ was evaluated in *Salmonella* tester strains TA98, TA100, TA1535, TA1537, and *E. coli* tester strain WP2uvrA in the presence and absence of an exogenous metabolic activation system (S9). The only activity observed for 2-OH-AQ were weak responses in strains TA100 and TA1537 and required the presence of S9 (Table 2).

Bacterial Mutagenicity of 9-Nitroanthracene (9-NA)

9-NA was evaluated for mutagenic activity in the *Salmonella* tester strains TA98 and TA100 in the presence and absence of an exogenous metabolic activation system (S9) Table 3). 9-NA tested positive in TA 98 minus S9 with a lowest observed effect level (LOEL) of 0.3 μg/plate and tested positive in TA100 with a LOEL of 10 μg/plate. No positive increases were observed in the presence of S9 at the concentrations used. The shape of the dose-response curve was not explored at higher concentrations because the compound was mutagenic at the lower doses relevant to the amount of contaminating material in the NTP cancer bioassay (Butterworth et al. 2001).

Mouse Lymphoma Assay with 9-Nitroanthracene (9-NA)

9-NA was evaluated for the ability to induce mutations in the L5178Y+/- mouse lymphoma forward mutation assay in the presence and absence of an S9 metabolic activation system. Range finding studies were conducted to identify doses used in the definitive assay (Materials and Methods). 9-NA was evaluated as negative in this assay in the absence of S9 (Table 4). 9-NA induced a positive mutagenic response in the presence of S9 beginning at doses as low as 5 μg/ml (Table 4).

In the mouse lymphoma assay small colony mutants carry chromosome aberrations associated with chromosome 11, the chromosome on which the TK locus is located in the mouse (Hozier et al. 1981). The positive response with 9-NA in the activation assay was primarily associated with an increase in small colonies suggesting a predominance of a clastogenic mechanism.

DISCUSSION

The primary purpose of the studies presented here was to provide additional information to help interpret the NTP bioassay with anthracene-derived AQ-OX (NTP, 2004). Reviewing the literature for this class of compounds showed that concerns with contamination extend to many studies with AQ and AQ derivatives. Anthracene is the starting material for AQ and several AQ derivatives. A predominant problem is that for a period of time the anthracene used was distilled from coal tar and different lots contained varying amounts of polycyclic aromatic hydrocarbon contaminants, particularly the mutagenic isomers of nitroanthracene (Cofrancesco 1992; Butterworth et al. 2001). Butterworth et al. (2001) identified 9-NA at 0.12% as the most prevalent contaminant in the NTP anthracene-derived AQ-OX bioassay material. The NTP reported a contaminant level in the bioassay material of 0.1% by GC analysis and of 0.5% using HPLC techniques with 9-NA as the most prevalent component. Because precise analytical information was needed in helping decide whether contaminating mutagenic and carcinogenic activity might reside with more than just the 9-NA, a new analytical study was undertaken.

The contaminating polycyclic aromatic hydrocarbons and nitroaromatic compounds that are often present in AQ-OX can present difficult challenges for purity analysis. Conventional analytical methods often fail to detect impurities of significant concern. An improved analytical procedure was specifically developed for the NTP AQ-OX in which the contaminants were removed and studied separately from the main AQ material. After most of the AQ had been removed by recrystallization, the remaining supernatant was quantitatively analyzed for contaminants. The results of this new analysis revealed that the contamination level in the AQ bioassay material was 0.65%. The individual component in the highest amount was 9-nitroanthracene at a level of 0.11%. Other classes included

polycyclic aromatic hydrocarbons at 0.09% (including anthracene, phenanthrene, and dibenzo (a,h) anthracene); nitrobenzene at 0.05% and unidentified organics and nitro-organics at 0.40%. From the perspective of the physical amount of contaminating material, it is possible that mutagenic and carcinogenic activity in the NTP anthracene-derived AQ-OX bioassay material may reside with more contaminants than just the 9-NA.

The biological activities of 1-OH-AQ and 2-OH-AQ are relevant because they are metabolites of AQ. Blomeke et al. (1992) reported that 1-OH-AQ was positive in the Ames mutagenicity bacterial tester strain TA1537 without S9. The NTP reported that 2-OH-AQ was a potent bacterial mutagen in strain TA98 also without S9 (NTP, 2004). However, neither 1-OH-AQ nor 2-OH-AQ appear to have the structure of a direct acting DNA-reactive mutagen and would not be expected to be mutagenic without metabolic activation. Once again the possibility must be addressed that such activity without S9 is actually being contributed by contaminants. Therefore, new mutagenicity studies were undertaken with purified 1-OH-AQ and 2-OH-AQ. The most revealing part of those studies was that no commercial preparation of 1-OH-AQ or 2-OH-AQ was found that did not contain substantial contaminating material (Materials and Methods). 1-OH-AQ and 2-OH-AQ were synthesized and purified in our laboratories using procedures that lowered chloroaromatic and nitroaromatic compound levels to below 5 ppm. In the bacterial mutagenicity assays, the only activity observed for 1-OH-AQ was a weak response in TA1537 and required the presence of S9 (Table 1). The only activity observed for 2-OH-AQ were weak responses in TA100 and TA1537 and required the presence of S9 (Table 2).

These observations suggest that the activity reported for 1-OH-AQ and 2-OH-AQ without S9 are due to contaminants (Blomeke et al. 1992; NTP, 2004). Thus, the comparative mutagenic potency calculations used by the NTP for these compounds

are likely not valid (NTP, 2004). It is not known why weak responses with S9 were seen with the 1-OH-AQ and 2-OH-AQ metabolites of AQ, while numerous genetic toxicology assays (all of which included metabolic activation) showed no activity at all with the parent compound AQ. It would appear that the amounts produced by metabolism and the resulting activity is simply too small to register in those assays with AQ.

Many nitroaromatic compounds are extremely potent mutagens and carcinogens (Pitts et al. 1982, Fu et al. 1985; Fu et al. 1986; Durant et al. 1996). Thus, the finding that 9-NA was a primary contaminant in the NTP anthracene-derived AQ-OX bioassay material raised several issues related to this class of compound. 9-NA tested positive in TA98 without S9 with a LOEL of 0.3 μg/plate and tested positive in TA100 with a LOEL of 10 μg/plate (Table 3). This pattern of activity without S9 is consistent with that seen with the anthracene-derived AQ-OX used in the NTP bioassay and suggests that 9-NA was responsible for at least part of the observed mutagenic activity. The low LOEL values observed are one indicator of potent mutagenic activity. The magnitude of the response, however, was not sufficient to account for all of the mutagenic response seen with the bioassay material (Butterworth et al. 2001). Therefore, the mutagenic activity noted in the bioassay material would appear to reside with more than just the 9-NA. This is consistent with the results of the analytical study presented above.

One interesting property of many of the nitroaromatic compounds is that they exhibit activity in bacterial mutagenesis assays without added metabolic activation. Examples of compounds with such activity include 9-NA, 2-NA, and 2-nitrofluorene.

The latter chemical is often used as a positive control in the Ames test without S9. These compounds, therefore, appear to in some way be either directly DNA reactive or are metabolized by the tester bacteria to reactive metabolites. Caution must be used in equating such activity in bacteria to activity in mammalian cells. Knowledge of activity in mammalian cells is more relevant in interpreting the potential influence of 9-NA on the NTP cancer bioassay. 9-NA exhibits mutagenic activity in a human cell line expressing P450 1A1 (Durant et al. 1996). In contrast to the bacterial assays, 9-NA was evaluated as negative in the mammalian cell mouse lymphoma mutagenicity assay in the absence of S9 (Table 4). However, 9-NA induced a positive mutagenic response in the presence of S9 beginning at doses as low as 5 μg/ml (Table 4). The positive response with 9-NA in the activation assay was primarily associated with an increase in small colonies suggesting a predominance of a clastogenic mechanism. This is particularly relevant because clastogens are frequently potent carcinogens.

While the NTP bioassay is directly applicable to exposure to anthracene-derived AQ-OX, the degree to which the contaminants may have influenced the induction of tumors is not known. Based on extensive testing of purified materials, AQ not a genotoxic carcinogen. Quantitative bacterial mutagenicity and carcinogenicity potency estimates indicate that it is plausible that the contaminants alone could have been responsible for all of the observed carcinogenic activity (Butterworth et al. 2001). The finding that the primary contaminant, 9-NA, is a potent mammalian cell mutagen and clastogen increases the possibility that the contaminants, rather than AQ produced the carcinogenic response.

These concerns have not been lost on those working in this area. The NTP recognized and addressed the contamination issue in their report (NTP 2004). Because of the numerous toxicology studies related to this issue, the coal tar based anthracene-derived AQ-OX production process is now seldom used. Industry pays a great deal of attention to sources and purity of AQ and AQ-OX is no longer commercially used in the United States. Analytical purity and verification are now more regular components of genetic toxicology testing protocols. The lingering legacy is, however, that many of the reported results in the literature for AQ and AQ derivative compounds need to be viewed with caution.

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TABLE 1

Activity of 1-OH-AQ in the *Salmonella* and *E. coli* Mutagenicity Assays

Without liver microsomes (S9)

1-OH-AQ	Average revertants per plate ± SD[a]				
μg / plate	TA98	TA100	TA1535	TA1537	WP2uvrA
0	14 ± 3	82 ± 12	10 ± 4	5 ± 2	14 ± 9
3.3	13 ± 5	65 ± 18	9 ± 3	10 ± 1	19 ± 4
10.0	15 ± 3	70 ± 4	12 ± 6	11 ± 2	17 ± 2
33.3	10 ± 2	80 ± 5	8 ± 3	11 ± 3	12 ± 3
100	14 ± 2	65 ± 3	11 ± 6	12 ± 5	14 ± 2
333	18 ± 4	77 ± 8	14 ± 3	8 ± 2	9 ± 3
1000	12 ± 2	71 ± 4	14 ± 2	16 ± 4	9 ± 1
pos. con.[b]	231 ± 17[a]	1114 ± 71[a]	694 ± 44[a]	1759 ± 157[a]	141 ± 42[a]

With liver microsomes (S9)

1-OH-AQ	Average revertants per plate ± SD[a]				
μg / plate	TA98	TA100	TA1535	TA1537	WP2uvrA
0	27 ± 8	101 ± 14	10 ± 2	9 ± 2	15 ± 4
3.3	24 ± 6	103 ± 13	10 ± 1	45 ± 7[a]	13 ± 3
10.0	25 ± 1	92 ± 11	11 ± 2	39 ± 7[a]	14 ± 2
33.3	19 ± 5	103 ± 18	17 ± 4	27 ± 7[a]	13 ± 3
100	26 ± 2	106 ± 1	12 ± 2	28 ± 1[a]	11 ± 3
333	19 ± 5	113 ± 8	12 ± 4	18 ± 7	14 ± 2
1000	22 ± 3	102 ± 15	13 ± 3	24 ± 3	15 ± 4
pos. con.[c]	481 ± 43[a]	953 ± 273[a]	144 ± 10[a]	188 ± 21[a]	351 ± 22[a]

[a] Judged as a positive response. Criteria for a positive response are an increasing dose response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a 3-fold increase for tester strains TA1535 and TA1537. A confirmatory study yielded approximately the same positive response pattern seen for TA1537 with S9.

[b] Positive controls without S9: TA98 1.0 μg 2-nitrofluorene; TA100 and TA1535 2.0 μg sodium azide; TA1537 2.0 μg ICR-191; WPuvrA 1.0 μg 4-nitroquinoline-N-oxide.

[c] Positive controls with S9: TA98 2.5 μg benzo[a]pyrene; TA100, TA1535, and TA1537 2.5 μg 2-aminoanthracene; WpuvrA 25 μg 2-aminoanthracene.

TABLE 2

Activity of 2-OH-AQ in the *Salmonella* and *E. coli* Mutagenicity Assays

Without liver microsomes (S9)

2-OH-AQ	Average revertants per plate ± SD[a]				
μg / plate	TA98	TA100	TA1535	TA1537	WP2uvrA
0	20 ± 6	85 ± 15	14 ± 2	9 ± 3	10 ± 0
1.0	15 ± 4	94 ± 2	13 ± 4	10 ± 1	ND
3.3	12 ± 2	88 ± 12	14 ± 4	11 ± 4	10 ± 2
10.0	16 ± 3	96± 7	13 ± 3	8 ± 2	9 ± 1
33.3	17 ± 3	86 ± 14	11 ± 1	8 ± 1	10 ± 3
100	12 ± 3	64 ± 5	9 ± 3	8 ± 3	8 ± 2
333	9 ± 2	10 ± 3	10 ± 2	13 ± 3	12 ± 6
1000	7 ± 2	0 ± 0	14 ± 2	13 ± 3	10 ± 5
pos. con.[b]	204 ± 14[a]	800 ± 96[a]	737 ± 85[a]	1150 ± 46[a]	136 ± 58[a]

With liver microsomes (S9)

2-OH-AQ	Average revertants per plate ± SD[a]				
μg / plate	TA98	TA100	TA1535	TA1537	WP2uvrA
0	24 ± 9	94 ± 12	11 ± 3	8 ± 3	12 ± 2
0.3	28 ± 9	113 ± 26	10 ± 4	21 ± 1	ND
1.0	29 ± 6	110 ± 21	13 ± 1	37 ± 3[a]	ND
3.3	24 ± 6	166 ± 13	13 ± 5	90 ± 7[a]	15 ± 7
10.0	35 ± 6	195 ± 13[a]	11 ± 1	114 ± 14[a]	16 ± 4
33.3	29 ± 5	177 ± 11	12 ± 4	99 ± 7[a]	10 ± 9
100	18 ± 2	61 ± 7	14 ± 2	53 ± 3[a]	10 ± 1
333	14 ± 3	0 ± 0	8 ± 2	35 ± 13[a]	10 ± 3
1000	ND	ND	ND	ND	13 ± 1
pos. con.[c]	462 ± 28[a]	1190 ± 103[a]	163 ± 12[a]	266 ± 62[a]	466 ± 73[a]

[a] Judged as a positive response. Criteria for a positive response are an increasing dose response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100, and WP2uvrA and/or at least a 3-fold increase for tester strains TA1535 and TA1537. A repeat study yielded approximately the same positive response pattern seen for TA 100 and TA1537 with S9.

[b] Positive controls without S9: TA98 1.0 μg 2-nitrofluorene; TA100 and TA1535 2.0 μg sodium azide; TA1537 2.0 μg ICR-191; WPuvrA 1.0 μg 4-nitroquinoline-N-oxide.

[c] Positive controls with S9: TA98 2.5 μg benzo[a]pyrene; TA100, TA1535, and TA1537 2.5 μg 2-aminoanthracene; WpuvrA 25 μg 2-aminoanthracene.

TABLE 3
Activity of 9-NA in the *Salmonella* Assay

Without liver microsomes (S9)

9-NA	Revertants/plate ± SD[a]	
μg / plate	TA98	TA100
0	15 ± 8	96 ± 10
0.1	25 ± 4	116 ± 1
0.3	31 ± 4[a]	92 ± 8
1.0	30 ± 9[a]	116 ± 6
3.0	37 ± 7[a]	137 ± 4
10.0	52 ± 1[a]	189 ± 8[a]
pos. con.[b]	385 ± 74[a]	1348 ± 33[a]

With liver microsomes (S9)

9-NA	Revertants/plate ± SD[a]	
μg / plate	TA98	TA100
0	24 ± 3	83 ± 18
0.1	27 ± 1	100 ± 7
0.3	31 ± 10	94 ± 1
1.0	27 ± 4	106 ± 25
3.0	32 ± 4	98 ± 28
10.0	38 ± 1	118 ± 2
pos. con.[c]	438 ± 43[a]	1397 ± 100[a]

[a] Judged as a positive response. Criteria for a positive response are an increasing dose response curve with at least one response equal to or greater than two times the mean vehicle control for tester strains TA98, TA100.

[b] Positive controls without S9: TA98 1.0 μg 2-nitrofluorene; TA100 2.0 μg sodium azide.

[c] Positive controls with S9: TA98 2.5 μg benzo[a]pyrene; TA100 2.5 μg 2-aminoanthracene.

TABLE 4

Activity of 9-NA in the L5178Y mutagenicity assay

9-NA minus S9 Concentration (μg/ml)	Relative Growth (%)[a]	Mutant Frequency (10E-6 units)[b]
0.0	100.0	53.7
0.1	86.8	68.0
1.0	99.6	61.7
10.0	101.0	60.7
20.0	32.7	58.3
30.0	6.4	84.1
Methylmethanesulfonate (MMS) positive control		
13 μg/ml	30.1	221.8[c]

9-NA plus S9 Concentration (μg/ml)	Relative Growth (%)[a]	Mutant Frequency (10E-6 units)[b]
0.0	100.0	82.0
0.1	43.2	90.8
1.0	59.7	82.3
5.0	25.0	176.4[d]
10.0	14.5	198.8[d]
50.0	5.3	321.8[d]
Methycholanthrene (MCA) positive control		
2 μg/ml	51.2	284.7[d]

[a] Relative Growth = (relative suspension growth x relative cloning efficiency) / 100.

[b] Mutant Frequency = (total mutant colonies/total viable colonies) x 2x10E-4. Decimal is moved to express the frequency in units of 10E-6.

[c] Mutagenic as judged by exceeding the minimum criterion of 107 for this experiment (two times the control frequency).

[d] Mutagenic as judged by exceeding the minimum criterion of 164 for this experiment (two times the control frequency).